Faculty of Resource Science and Technology

DETECTION AND ENUMERATION OF *BACILLUS CEREUS* IN FORMULA MILK AND ULTRA HIGH TEMPERATURE (UHT) TREATED MILK PRODUCTS

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Detection and Enumeration of *Bacillus cereus* in Formula Milk and Ultra High Temperature (UHT) Treated Milk Products

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A Thesis submitted in partial fulfillment of
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LIST OF ABBREVIATIONS

BAM	Bacteriological Analytical Manual
B. cereus	*Bacillus cereus*
bp	base pair
CDC	Centre for Disease Control and Prevention
cfu	Colony-forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
g	gram
gyrB	gyrase B
h	hour
HTST	High-temperature short-time
IDACE	European Dietetic Food Industry Association
ISO	International Organization for Standardization
min	minute
MPN	Most probable number
$MgCl_2$	Magnesium chloride
PCR	Polymerase chain reaction
RTE	Ready-to-eat

SNE	Specialised Nutrition Europe
TBE	Tris-boric-EDTA
UHT	Ultra high temperature
V	Volt

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Detection and Enumeration of *Bacillus cereus* in Formula Milk and Ultra High Temperature (UHT) Treated Milk Products

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ABSTRACT

Bacillus cereus (*B. cereus*) is an important food borne pathogen that is widely distributed in nature. *B. cereus* has been isolated from many foods including dairy products, vegetables, meat and rice which often lead to outbreaks occurring in the world. Presence of *B. cereus* in milk products such as formula milk and UHT milk is of particular concern considering the majority of consumers are infants and children. The aim of this study was to enumerate and detect the presence of *B. cereus* in both formula milk (n=12) and UHT milk (n=20) sold in retail markets in Malaysia by employing a combination of MPN and PCR approach. Of all the samples tested, 41.67% (5/12) and 30% (6/20) were detected with *B. cereus* in formula milk and UHT milk respectively. Contamination level of *B. cereus* in formula milk ranged from < 3 to > 1100 MPN/g. Similarly, the level of contamination was also in the range of < 3 to > 1100 MPN/mL in UHT milk samples. The contamination level of *B. cereus* was found to be highest in full cream UHT milk and young-child formula. Therefore, it is crucial for the health and food safety authorities to address this issue as higher contamination level may be present in similar milk products.

Keywords: *Bacillus cereus*, MPN-PCR, UHT, milk, formula

ABSTRAK

Bacillus cereus (B. cereus) merupakan patogen bawaan makanan yang penting dan terdapat dalam alam semula jadi secara meluas. Bakteria ini telah diisolasikan dari pelbagai jenis makanan termasuk produk tenusu, sayur-sayuran, daging dan nasi yang mungkin menyumbang kepada wabak yang berlaku di dunia ini. Kemunculan B. cereus dalam susu formula dan susu UHT harus diperhatikan kerana kebanyakan pengguna adalah bayi and kanak-kanak. Matlamat kajian ini adalah untuk mengetahui tahap kontaminasi dan mengenalpasti identiti B. cereus yang terdapat dalam susu formula (n=12) dan susu UHT (n=20) yang dipasarkan di Malaysia dengan menggunakan cara kombinasi MPN dan PCR. Dalam semua sampel yang telah diperiksa, 41.67% (5/12) dan 30% (6/20) adalah positif untuk B. cereus dalam susu formula dan susu UHT masing-masing. Konsentrasi B. cereus dalam susu formula adalah dalam lingkungan < 3 hingga > 1100 MPN/g manakala susu UHT juga menunjukkan konsentrasi dalam lingkungan < 3 hingga > 1100 MPN/mL. Tahap kontaminasi B. cereus adalah tertinggi dalam susu penuh krim dan susu kanak-kanak. Oleh itu, kajian ini adalah penting untuk pihak berkuasa untuk mengendali isu ini kerana tahap kontaminasi yang lebih tinggi mungkin muncul dalam produk susu yang lain.

Kata kunci: Bacillus cereus, MPN-PCR, UHT, susu, formula

1. INTRODUCTION

1.1 Introduction

Food borne diseases have devastating health and economic impacts to the world. Based on a report by European Food Safety Authority (EFSA) in 2005, 1 to 33% of food borne poisoning was attributed to *Bacillus cereus* (*B. cereus*) (Sandra *et al.*, 2012). An increment of 122.2% in food poisonings cases caused by *B. cereus* in the Europe were reported to the EFSA in the year 2011 (Messelhäusser *et al.*, 2014).

B. cereus is a well-known etiological agent of food borne illness responsible for two different types of gastrointestinal disorders: emetic syndrome and diarrheal syndrome (Magni, 2008; Messelhäusser *et al.*, 2014; Rahimi *et al.*, 2013). The emetic syndrome is characterized by nausea and vomiting after short period of time upon consumption of contaminated food (Messelhäusser *et al.*, 2014; Sadek *et al.*, 2006). The symptoms exhibited are often mild and also similar to intoxication caused by *Staphylococcus aureus*. Diarrheic poisoning is associated with abdominal pain (Shinagawa, 1990) and watery diarrhea (Andreoletti *et al.*, 2013). Due to its mild symptoms, food intoxication implicated by *B. cereus* may risk from misdiagnosis and is frequently underreported (Messelhäusser *et al.*, 2014). However, fatality linked to *B. cereus* had also been reported due to the consumption of contaminated pasta (Dierick *et al.*, 2005).

B. cereus is a Gram positive and facultative anaerobic, rod-shaped bacterium (Montanhini and Bersot, 2013; Tunio *et al.*, 2013). The organism is a common soil-dweller (Lee *et al.*, 2010; Park *et al.*, 2007) and has the capability to form endospores which allows it to survive in extreme environmental conditions. Due to their resistant endospores, they can thrive in various food processing procedures such as drying and heat treatment

(Rosenquist *et al.*, 2005; Tunio *et al.*, 2013). *B. cereus* has been reported in various foods such as dairy products, rice, vegetables and meat (Lee *et al.*, 2010; Manzano *et al.*, 2003; Tunio *et al.*, 2013). According to Becker *et al.* (1994), contamination by *B. cereus* was often found in dried milk products and infant foods.

Food poisoning cases associated with milk-based products have been reported and as high as 85% of enterotoxigenic *B. cereus* have been isolated from milk and milk products (Sadek *et al.*, 2006). Occurrences of *B. cereus* in milk products are especially important in the baby formula industry. Infants are more susceptible to food borne infections due to under-developed immune system (Drudy *et al.*, 2006; Food Standards Australia New Zealand, 2004; Tunio *et al.*, 2013) and absence of competing microorganisms in gut microflora (Tunio *et al.*, 2013). Moreover, infant and toddler milk powder often contain raw ingredients from various sources and are rich in nutrients. When reconstituted and left at ambient temperatures for longer periods, these milk products will become a suitable medium for proliferation and enterotoxin production of *B. cereus* (Tunio *et al.*, 2013). Hence, frequent exposure of infants and toddlers to these milk products increases the risk of contracting food borne illness. Recall of infant feeding products has been reported upon development of gastrointestinal disorders in infants which may be caused by microbial contamination (Samakow, 2012; Walker, 2010).

Incidences of *B. cereus* in processed milk products have also been reported. *B. cereus* spores are known to survive pasteurization (Grande *et al.*, 2006) and has been isolated from ultra high temperature (UHT) treated milk (Ubong *et al.*, 2013). An outbreak associated with *B. cereus* in contaminated UHT milk was also reported which had affected 191 students from Malaysia (Mhd Yusof, 2011). In the year 2011, a recall for Australian UHT skim milk had been reported which was due to microbial spoilage (FSANZ, 2014).

According to the European Dietetic Food Industry Association (IDACE), the proposed safety limit of *B. cereus* in food is 10^3 cfu ml^{-1} (Rowan and Anderson, 1998). Consumption of milk exceeding the regulatory limits may pose a health hazard to the consumers as 10^5 to 10^6 cells or spores/g of food can cause food poisoning (Soleimaninanadegani, 2013). Lower infectious dose may also lead to illness especially in potentially fragile consumers. Therefore, the aim of this study was to determine the prevalence of *B. cereus* in formula milk and UHT milk by adopting MPN method in combination with PCR.

1.2 Objectives

The objectives of this study were to:

1. Determine the level of *Bacillus cereus* present in formula milk and UHT milk by using the most probable number (MPN) method.
2. Identify the presence of *B. cereus* in formula milk and UHT milk by performing polymerase chain reaction (PCR).

2. LITERATURE REVIEW

2.1 Taxonomy and Characteristics of *Bacillus cereus*

The genus *Bacillus* belongs to the family Bacillaceae (Rukure, 1999). *B. cereus* taxonomically belongs to the genus *Bacillus* and is grouped under the *B. cereus* group which comprises of six closely related species: *B. cereus, Bacillus anthracis, Bacillus weihenstephanensis, Bacillus pseudomycoides, Bacillus mycoides,* and *Bacillus thuringiensis* (Lin, 1997; Noonan, 2014). Differentiation and identification between these *Bacillus* species is difficult due to their close genetic and phenotypic relationship (Bottone, 2010; Park *et al.*, 2007). Phenotypic differences between members of *B. cereus* group are mainly due to the presence of extrachromosomal plasmids and often require molecular approaches for identification (Noonan, 2014). *B. cereus* has two distinct morphological appearances, as vegetative cell or endospore (A. Abd *et al.*, 2010). When as vegetative cell, *B. cereus* is facultatively anaerobic with a rod-like shape (Bottone, 2010; Němečková *et al.*, 2011; Park *et al.*, 2007) and grows to a size of 1.0-1.2 μm × 3.0-5.0 μm (A. Abd *et al.*, 2010). *B. cereus* vegetative cells are Gram positive rods which tend to form long chains (A. Abd *et al.*, 2010). Even though they are Gram positive, they can also be Gram-variable or Gram negative during the later stage of cell growth (A. Abd *et al.*, 2010; Noonan, 2014). The endospores are ellipsoidal or cylindrical which are located centrally or paracenter positions that do not distort the bacillary shape or cause swelling of sporangia (Lin, 1997; Noonan, 2014).

B. cereus can thrive in a wide range of environmental stress such as extreme temperature and pH (Rowan and Anderson, 1997). They can grow at temperatures between 4 °C and 55 °C (Magni, 2008) with an optimum growth at 30 - 37 °C. The range of pH that allows cell growth is between pH 4.5 and pH 9.3 (Magni, 2008; Rukure, 1999). Spore-forming ability of *B. cereus* allows them to survive in adverse conditions. The spores are heat-resistant and can tolerate from pH 1.0 to pH 5.2 (Magni, 2008). According to Magni (2008) and A. Abd *et al.* (2010), toleration of salt concentration in *B. cereus* is as high as 7.5%.

2.2 Pathogenicity of *Bacillus cereus*

B. cereus is capable of producing toxins which is the main cause of two types of food poisoning: emesis and diarrhea (Magni, 2008; Rosenquist *et al.*, 2005; Soleimaninanadegani, 2013). The emetic syndrome is due to the heat-stable cereulide which can withstand extreme pH from pH 2 to pH 11 (Lin, 1997) such as in stomach acid (Linbäck and Granum, 2006) and resist proteolysis (Finlay *et al.*, 2000) in the intestinal tract. Production of this toxin is during the late exponential or stationary phase (Lin, 1997) of the vegetative cells in foods (Linbäck and Granum, 2006; Rosenquist *et al.*, 2005). Death of the cells would not result in the destruction of this toxin (Magni, 2008). Symptoms triggered by this toxin include vomiting (Linbäck and Granum, 2006), nausea, abdominal cramps and diarrhea (Andreoletti *et al.*, 2013). The diarrhea syndrome is caused by another different heat-labile enterotoxins formed in food (Di Pinto *et al.*, 2013) or produced in the small intestine by the ingested bacteria (Linbäck and Granum, 2006). The three different enterotoxins involved in causing the illness are haemolysin BL (HBL), non-haemolytic enterotoxin (NHE) and cytotoxin K (Cyt K). These toxins are produced during the late log phase of the cells (Lin, 1997; Magni, 2008). Destruction and inactivation of these toxins can be done by cooking at 56 °C for 5 minutes or exposing them to pH below 4 or above 11 (Lin, 1997). These toxins can induce watery diarrhea (Andreoletti *et al.*, 2013) and abdominal pain (Shinagawa, 1990). According to Magni (2008), consumption of food that contains cells or spores exceeding 10^4/g pose a risk in the development of diarrhea syndrome. However, the number of *B. cereus* can range from 10^5 to 10^9/g for emetic syndrome (Magni, 2008).

2.3 Occurrences of *Bacillus cereus* in Food

B. cereus is ubiquitous in nature and can be commonly isolated from diverse food matrices (Rosenquist *et al.*, 2005). Spores of *B. cereus* have been found in various types of cereals, pulses, vegetables, spices (CDC, 1986; Dikbas, 2010), and dried milk products (CDC, 1986; Di Pinto *et al.*, 2013; Grande *et al.*, 2006). However, foods that are linked to intoxication are often rice-based products, pasta and noodles. Contamination level exceeding 10^3 cfu/g of *B. cereus* was also reported in cereal products and in rice (cooked and uncooked) (Grande *et al.*, 2006).

Many studies have been conducted to determine the occurrences of *B. cereus* and related microorganisms in food. Ready-to-eat (RTE) foods are among one of the most intensively studied subject. A study by Rosenquist *et al.* (2005) reported that 0.5% from a total of 48, 901 samples of RTE food products in Denmark were positive for *B. cereus*-like bacteria with counts exceeding 10^4 cfu/g. These RTE foods ranged from fresh food (lettuce, fruits, cucumbers, etc.) to heat-treated food (sauces, soups, pasta, bread, etc.) or a combination of both. The higher counts of these bacteria were found in starch, cooked products as well as fresh cucumbers and tomatoes (Lesley *et al.*, 2013; Rosenquist *et al.*, 2005). Similarly, in Malaysia, RTE foods from 33 school hostel kitchens and canteens collected over a period of six months were examined and the results showed that 1.9% (5/264) of RTE foods were contaminated by *B. cereus* (Jeyaletchumi *et al.*, 2006).

Growing popularity of RTE cereals in consumers' breakfast diet is evident especially in Malaysia (Lesley *et al.*, 2013). In a study by Lee *et al.* (2009), 78% of RTE cereals were contaminated by *B. cereus* and the concentration ranged from 30 to more than 24, 000 MPN/g. Another study by Daczkowska-Kozon *et al.* (2009) also revealed the high percentage of *B. cereus* group bacteria found in retail packed cereals. Frequency of *B. cereus* isolated from wheat cereals was 100%, followed by buckwheat (85.7%) and pearl barley (85%). Most of the tested positive samples showed contamination level exceeding 10^2 MPN/g (Daczkowska-Kozon *et al.*, 2009).

Other than RTE foods and cereals, *B. cereus* was also isolated from tropical seafood. Sixty-eight samples including fish, shrimps and clams were tested for the presence of enterotoxigenic *B. cereus*. It was reported that 25 samples were positive for *B. cereus* whereby enterotoxigenic *B. cereus* were isolated from 29.41% of fish samples (Das *et al.*, 2009).

Presence of *B. cereus* in dairy products is a concern in food production industry as this bacterium is responsible for food spoilage, emesis, diarrhea and fatal meningitis (Rahimi *et al.*, 2013). According to Montanhini *et al.* (2013), *B. cereus* were isolated from various dairy products such as raw milk, pasteurized milk, UHT milk, ice-cream, milk powder, and fermented milk. *B. cereus* was also found in 17.6% (15/85) of refrigerated dairy products in a study by Montanhini *et al.* (2013). Another class of dried milk product of concern is infant foods as increasing trend of contamination by *B. cereus* predominantly with its spores was reported (Di Pinto *et al.*, 2013). High contamination rate of infant formulae, follow-up formulae and weaning foods were reported in a study by Becker *et al.* (1994). Fifty-four percent of the examined infant foods and dried milk products were found to be contaminated with *B. cereus* with levels reaching up to 600 *B. cereus*/g. Recent studies in several nations also showed high prevalence of *B. cereus* in infant foods. Rahimi *et al.* (2013) showed that 42% (84/200) of Iranian infant foods were contaminated by *B. cereus*. Di Pinto *et al.* (2013) also reported that 5 out of 11 samples of powdered infant formula contained *B. cereus*. Findings of Tunio *et al.* (2013) also revealed that infant formula milks and powdered protein-based shakes in Pakistan were contaminated by *B. cereus*.

2.4 Most Probable Number (MPN) Method

MPN is a statistical method frequently used in food microbiology (Oblinger and Koburger, 1975; Woomer *et al.*, 1990) and environmental monitoring (Sutton, 2010). It is based on the probability theory whereby the samples are serially diluted to a point where there is no more viable microorganism. Inoculation of these serial dilutions into appropriate growth medium allows the detection of end point. Growth of bacteria can be indicated by examining the turbidity of the tubes thus statistical probability tables are used to determine the concentration of the bacteria in the original sample. Replicates of 3, 5 or 10 tubes for each dilution are normally used and referred to the statistical MPN table (Atlas *et al.*, 1988). As more replicates are used, accuracy of the method increases (Sutton, 2010; Woomer *et al.*, 1990) thus giving a more precise estimate of the bacterial population size (Atlas *et al.*, 1988).

There are a few assumptions to be made for this method. Firstly, it assumes the distribution of microorganisms throughout the sample to be random (Blodgett, 2010) and serial dilutions done are accurate. This would mean that the microorganisms are freely apart and are not affected by each other. Secondly, it assumes that each inoculum from each tube would contain at least a single viable microorganism that leads to visible growth in suitable growth medium (Blodgett, 2010; Oblinger and Koburger, 1975; Sutton, 2010).

The use of contaminant-free equipments and supplies is also assumed as well as certain degree of technical skills is involved (Oblinger and Koburger, 1975).

MPN technique is widely adopted in many food testing protocols to enumerate different species of food borne pathogen. MPN method can be more advantageous over standard plate count method. Its higher sensitivity allows detection of lower numbers of bacteria (<100/g) in food especially milk (Blodgett, 2010). Enumeration with this technique is less precise as there is no upper boundary of the concentration if all tubes are positive for every dilution (Blodgett, 2010; FSANZ, 2004). In contrast, the detection limit of standard plate count method is 100 CFU/g (FSANZ, 2004), thus MPN would be a better procedure to detect low levels of bacteria occurring in the food. This is supported by Harper *et al.* (2011) in their study in comparing the effectiveness of plating method and MPN method to enumerate *B. cereus* spores in raw and pasteurized milk. They recovered more *B. cereus* using the MPN method instead of plate count using mannitol-egg yolk-polymyxin agar. The overall mean population of *B. cereus* of pooled sampling times is significantly greater in MPN method than plate count (Harper *et al.*, 2011). In addition, studies using MPN method for enumeration of *B. cereus* in milk products had been established. Zhou *et al.* (2008) studied the occurrence of *B. cereus* and related organisms in pasteurized full fat milk. They showed that an average of 11.7 MPN/mL of *B. cereus* was found in the positive samples. Ubong *et al.* (2013) also used MPN method to enumerate *B. cereus* in UHT milks which showed that the contamination level ranged from < 3 to 11 MPN/mL.

3. MATERIALS AND METHODS

The materials used in this study are listed in Appendix I.

3.1 Sample Collection

A total of 20 samples of one liter UHT milk from different manufacturers were purchased from the local supermarkets and hypermarkets in Kuching and Kota Samarahan, Sarawak. Twelve samples of formula milk including infant formula, follow-up formula and young-child formula were donated by residents of Kuala Lumpur and Kuching, Sarawak. The details of the products were shown in Table 1 and Table 2.

Table 1. List of formula milk samples collected from Sarawak and Kuala Lumpur

Abbreviation	Product Type	Product Name	Place of Collection
F1	Young-child formula	Dumex Mamil Learning 3	Kuching, Sarawak
F2	Infant formula	Nestle Lactogen 1 Infant Formula	Kuching, Sarawak
F3	Follow-up formula	Snow'brand Neo Baby Step 2	Ampang, Kuala Lumpur
F4	Infant Formula	Abbott Isomil Rumusan Khas Soya Tanpa Laktosa	Kepong, Kuala Lumpur
F5	Follow-up formula	Bonmil Formulated Organic Milk	Ampang, Kuala Lumpur
F6	Young-child formula	Pediasure® Complete	Ampang, Kuala Lumpur
F7	Young-child formula	Snow brand Neo-Kid Plus Step 3	Ampang, Kuala Lumpur
F8	Young-child formula	Dutch Lady Growing Up Milk	Kepong, Kuala Lumpur
F9	Young-child formula	Anmum Essential Growing Up Milk Powder Step 3	Kuching, Sarawak
F10	Young-child formula	Similac Gain Kid Intelli-Pro Step 4	Kepong, Kuala Lumpur
F11	Infant formula	Dumex Dulac	Summer Mall, Sarawak
F12	Follow-up formula	Dumex Dupro	Summer Mall, Sarawak

Table 2. List of UHT milk samples purchased from Kuching and Kota Samarahan, Sarawak.

Abbreviation	Brand Name	Product Name	Date of Purchase	Place of Purchase
U1	Harvey Fresh	Lite Milk	November 1, 2014	Everrise, Desa Ilmu
U2	Meadow Fresh	Pure Milk Full Cream	September 27, 2014	H&L Supermarket, Batu 7
U3	Marigold	Full Cream Milk	November 1, 2014	Servay Hypermarket, Summer
U4	Harvey Fresh	Full Cream Milk	October, 22, 2014	Everrise, Padungan, Kuching
U5	Cowhead	Pure Milk	October, 22, 2014	Everrise, Padungan, Kuching
U6	Meadow Fresh	Pure Milk Low Fat	December 13, 2014	The Spring, Kuching
U7	Dutch Lady	Full Cream Milk	September 27, 2014	H&L Supermarket, Batu 7
U8	Dutch Lady	Low Fat High Calcium Milk	November 1, 2014	Servay Hypermarket, Summer
U9	Dutch Lady	Fresh Milk	November 1, 2014	Servay Hypermarket, Summer
U10	Daily Dairy	UHT Recombined Full Cream Milk	September 27, 2014	H&L Supermarket, Batu 7
U11	Emmi	Swiss Premium Milk	December 13, 2014	The Spring, Kuching
U12	Devondale	Full Cream Milk	December 13, 2014	The Spring, Kuching
U13	Marigold	Low Fat Milk	November 1, 2014	Servay Hypermarket, Summer
U14	Devondale	Skim Milk	December 13, 2014	The Spring, Kuching
U15	Nestlé	U.H.T Recombined Milk- Low Fat	November 1, 2014	Servay Hypermarket, Summer
U16	Anchor	Full Cream Milk	December 13, 2014	The Spring, Kuching